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Appendage-Mediated Surface Adherence of *Sulfolobus solfataricus*▽

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Attachment of microorganisms to surfaces is a prerequisite for colonization and biofilm formation. The hyperthermophilic crenarchaeote *Sulfolobus solfataricus* was able to attach to a variety of surfaces, such as glass, mica, pyrite, and carbon-coated gold grids. Deletion mutant analysis showed that for initial attachment the presence of flagella and pili is essential. Attached cells produced extracellular polysaccharides containing mannose, galactose, and *N*-acetylglucosamine. Genes possibly involved in the production of the extracellular polysaccharides were identified.

In microbiology, organisms are isolated from their natural habitats and typically cultivated in the laboratory as planktonic species. Though this method has been essential for understanding the concept of life, it remains unclear how microbial ecosystems operate. For bacteria, it is well known that they are able to form large cellular communities with highly complex cellular interactions and symbioses between different microbial or eukaryotic species. Biofilm formation is an essential component of such communities, and studies have shown that bacteria within biofilms are physiologically different from planktonic ones (20, 21). They can exhibit extensive networks of pili on their surfaces and produce and secrete extracellular polysaccharides (EPS), their growth rate is decreased, and cells are much more resistant to physical stresses and antibiotics (19).

The study of surface colonization and cellular communities of archaea is crucial for understanding their ecological properties. The only detailed study showed that the hyperthermophilic organism *Archaeoglobus fulgidus* produced biofilms when challenged with heavy metals and pentachlorophenol (10). *Pyrococcus furiosus* was able to adhere to different surfaces, such as mica and carbon-coated gold grids, and cells were connected via cable-like bundles of flagella (12). *Methanopyrus kandleri* was shown to adhere to glass, but *P. furiosus* could colonize only by attaching to *M. kandleri* cells, using flagella and direct cell contacts (16).

Here we report on the function of cell surface appendages in initial attachment to surfaces of archaea, using directed gene inactivation mutants. The crenarchaeote *Sulfolobus solfataricus* P2 is a thermoacidophile which grows optimally at 80°C and pH values of 2 to 4 (22). *S. solfataricus* possesses cell surface structures such as flagella and UV-induced pili (1, 2). The flagellum operon of *S. solfataricus* encodes, in addition to the structural subunit FlaB, four proteins of unknown function, the ATPase FlaI, and the only integral membrane protein, FlaJ. Previously, we isolated a Δ*flaJ* mutant which was nonflagellated and had lost its ability for surface motility on Gelrite plates (17). Recently, we described UV-inducible pili in *S. solfataricus* that directed cellular aggregation after UV stress (8). Deletion of the central ATPase UpsE, responsible for pilus assembly, rendered cells devoid of pili and defective in cellular aggregation after UV treatment (8). In this study, wild-type cells and deletion strains were tested for the ability to attach to a variety of surfaces and the formed structures and extracellular material were analyzed.

MATERIALS AND METHODS

Strains and growth conditions. *S. solfataricus* P2, *S. solfataricus* PBL2025 (15), and Δ*flaJ* (17) and Δ*upsE* (8) mutants were grown aerobically at 80°C in the medium described by Brock et al. (5), adjusted to pH 3 with sulfuric acid, and supplemented with 0.1% (wt/vol) tryptone under moderate agitation. Growth of cells was monitored by measuring the optical density at 600 nm.

Sample preparation for electron microscopy. Samples on 200-mesh carbon-coated gold grids were negatively stained with 2% uranyl acetate and analyzed by transmission electron microscopy on a Philips CM12 electron microscope (LaB6 cathode, 120 keV; FEI Co., Eindhoven, The Netherlands). Samples for scanning electron microscopy were freeze-dried for 2 h at −80°C (CFE 50; Cressington Ltd., Watford, United Kingdom), rotary shadowed with platinum-carbon (1 to 2 nm), and analyzed with an FEI Quanta 400 scanning electron microscope (FEG cathode; 4 to 25 keV).

Fluorescence microscopy. Cells were grown for 3 days in 0.1% tryptone medium in the presence of a glass slide. After cooling down of the culture, the cells were fixed with 4% formaldehyde for 30 min. The glass slides were washed with Brock medium (pH 5) to remove planktonic cells. Cells on the lower side of the glass slides were removed with ethanol. Lectins were applied to the glass slides, evenly spread with Parafilm, and incubated for 30 min at room temperature in the dark. Fluorescently labeled lectins used were concanavalin A (ConA)-fluorescein conjugate (1 mg/ml), GS-II–Alexa Fluor 594 from *Griffonia simplicifolia* (1 mg/ml), and GS-IB4–Alexa Fluor 594 from *G. simplicifolia* (1 mg/ml). Unbound lectins were removed by washing with Brock medium (pH 5). All lectins were obtained from Molecular Probes. The samples were analyzed by fluorescence microscopy, using a red-filter 750-ms exposure and green-filter 250-ms exposure.

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TABLE 1. Primers used for RT-PCRs and qPCRs

Primer	Sequence (5′–3′)
UpsA-rt-forward	GCTGGGTGGTCTACTTTAT
UpsA-rt-reverse	AGTACTGCCCAGCAGTTA
UpsA-rt-forward	TCTCACTCATCGTTCCATTA
UpsA-rt-reverse	CAGAGTTTCCCTCAATGAAT
FlaB-rt-forward	AGACAGCGTCAACAGACTA
FlaB-rt-reverse	ACCTGCACTTGTCTGCTGAT
sso3002-rt-forward	TGTTGGAGATGGGTGTGATG
sso3002-rt-reverse	AGCTGGGTCTGTCGTAATTG
sso3012-rt-forward	CCCAACTTCCTCGTTGTTAG
sso3012-rt-reverse	TGCCCTCGCAATACTTAACC
sso3042-rt-forward	TTTGCGGTACTGATAGGGAG
sso3042-rt-reverse	CTGAAGCGCCTGTAGTATCG
sso3043-rt-forward	TACTTCACTGACGGCACACC
sso3043-rt-reverse	CCGTGGGATTCAAGCAACTG
SSO3041-rt-forward	CTGGTCTGCCTGGTTCATAC
SSO3041-rt-reverse	CACATCCCTCTGCCTTATTG
SSO3039-rt-forward	GAGAAGACTACGCAGTTCCA
SSO3039-rt-reverse	GCGTTCCTAGCAACCTCATA
SSO3036-rt-forward	CCTATGGGCACCTAAAGGTA
SSO3036-rt-reverse	CTGCAACCTCACGTATATCG
SSO302-rt-forward	ACTCTAAAGGTCGCTGAGTG
SSO3021-rt-reverse	GGAAATTGGCTTGCCTCTTG
SSO3017-rt-forward	GGGTGAGTAGGAATAGTAGG
SSO3017-rt-reverse	GGAATCAGATAGGGCGTAAG
SSO3010-rt-forward	GCGAACTTCGGTTGGTTACT
SSO3010-rt-reverse	TACCCACGAGCCTCTGTAAT
SSO3007-rt-forward	ATATGATAGGAGCGCGGGAT
SSO3007-rt-reverse	CCCTACTTCCTGCTGGATTA
SSO3014-rt-forward	CCAGACCCAGATACTCCAAA
SSO3014-rt-reverse	CAACATCCTTGAGGCCTAAC
SSO3038-rt-forward	TTAGGCGTGTAGGAGGACAA
SSO3038-rt-reverse	ACCAGCCATCTATCCCTGAA
secY-rt-forward	GGATCGGGAGTTAGTCTGTT
secY-rt-reverse	GAAGCTGAGGGTGAGACATA

Gene expression analysis. *S. solfataricus* P2 cultures were incubated with pieces of mica for 2 days. Total RNAs were then isolated from planktonic cells and cells attached to the mica. Total RNA isolation and cDNA synthesis were performed as described previously (23). Gene-specific primer sets (Table 1) were used to detect the presence of selected genes in the SSO3002-3050 genomic region. PCR products were analyzed on 0.8% agarose gels.

Quantitative PCR (qPCR) analysis was carried out according to the protocol and chemicals provided by Applied Biosystems. For each gene of interest, a duplicate setup of 26 μl PCR mixture was prepared from 13 μl Sypro green master mix, 2 μl of a 5 μM primer pair stock solution, 2 μl cDNA, and 9 μl nucleotide-free water. The negative control assays were done with RNA mixtures that were prepared for cDNA synthesis. Primer efficiencies were calculated from the average slope of the linear regression curves according to the calculation model advised by Applied Biosystems. The fluorescence quantities of the reactions were measured with an ABI 7500 instrument (Applied Biosystems, Foster City, CA).

RESULTS

Attachment of *S. solfataricus* to various surfaces. For initial attachment to different materials, we tested *S. solfataricus* P2, *S. solfataricus* PBL2025, and the Δ*flaJ* and Δ*upsE* mutants derived from this strain. Carbon-coated gold grids were incubated in shaking cultures for 2 days in tryptone medium. PBL2025 adhered to the carbon-coated gold grids, and some flagella and more straight pili were present (Fig. 1A and C). In contrast, only very few cells of the Δ*flaJ* (Fig. 1B) and Δ*upsE* (Table 2) strains attached to the carbon grids, implying an important role for both the flagella and UV-induced pili for this process. The same experiment was repeated with the addition of sulfur to the tryptone medium to mimic the natural habitat of *Sulfolobus* species. Considerably more PBL2025 cells were found attached to the carbon film than to tryptone medium alone (Fig. 1D). Moreover, the cells clustered around the sulfur particles (Fig. 1E) and developed some extracellular sheet-like structure connecting the cells (Fig. 1F). Other materials tested for attachment of cells were glass, pyrite, and mica, a layered aluminum silicate which forms extremely smooth and clean surfaces when cleaved with a razor blade (for an overview, see Table 2). *S. solfataricus* P2 and PBL2025 grew very differently on mica surfaces. PBL2025 formed microcolonies and produced very large, thin layers of extracellular material in which cells were embedded (Fig. 2A and B). *S. solfataricus* P2 formed a more regular, extended network of extracellular appendages containing some other material between the structures (Fig. 2C and D). As shown for carbon-coated grids, cells of the Δ*flaJ* and Δ*upsE* strains were also unable to adhere to mica (data not shown). *S. solfataricus* P2 grew best on glass surfaces, yielding more cells than on mica or carbon-coated grids. Obviously, the cells attached to the surfaces by using flagella and pili (Fig. 2E and F), but they did not produce the extensive network of extracellular material and appendages observed on mica. The Δ*flaJ* and Δ*upsE* strains were also not able to grow on glass (Table 2).

Analysis of extracellular material from attached cells. It is well known that extracellular proteins of *Sulfolobales* are glycosylated. Glucose, mannose, galactose, and *N*-acetylglucosamine have previously been identified in the glycans of sugar-binding proteins (7). Moreover, the same sugars were found in extracellular polysaccharides that are produced mainly in the stationary growth phase of *S. solfataricus* MT3 and MTA4 (13). Therefore, fluorescently labeled lectins were used to determine the nature of the extracellular material produced by the attached *S. solfataricus* cells. Glass slides were incubated for 3 days in shaking *S. solfataricus* P2 and PBL2025 cultures. After fixation, the samples were incubated with fluorescently labeled lectins directed against terminal α-D-galactosyl residues (isolectin-IB4), α- or β-linked *N*-acetyl-D-glucosamine (GS-II), or α-mannopyranosyl and α-glucopyranosyl residues (ConA). In *S. solfataricus* P2, all three lectins reacted with the extracellular material, indicating the presence of the different sugars recognized by the different lectins (Fig. 3A to H). ConA and isolectin-IB4 also bound to cells staining the cell envelope, whereas GS-II only attached to the extracellular material. As shown by the scanning electron images (Fig. 2), PBL2025 formed a denser-appearing extracellular material, which was also visible by light microscopy (Fig. 3I to P). Also with PBL2025, all three lectins bound to the extracellular material; interestingly, and in contrast to the case with *S. solfataricus* P2, the lectin GS-II also bound to the cell envelope. From this analysis, it can be concluded that *Sulfolobus* strains produce EPS when attached to a glass surface.

Analysis of differentially expressed genes in attached *S. solfataricus* P2 cells. As shown in Fig. 2, attachment of *S. solfataricus* P2 and PBL2025 to mica resulted in diverse structures; in particular, the extracellular material formed had a different appearance. In contrast to *S. solfataricus* P2, strain PBL2025 lacks genes SSO3004 to -3050 (15). This region includes a diverse set of genes possibly involved in sugar degradation and

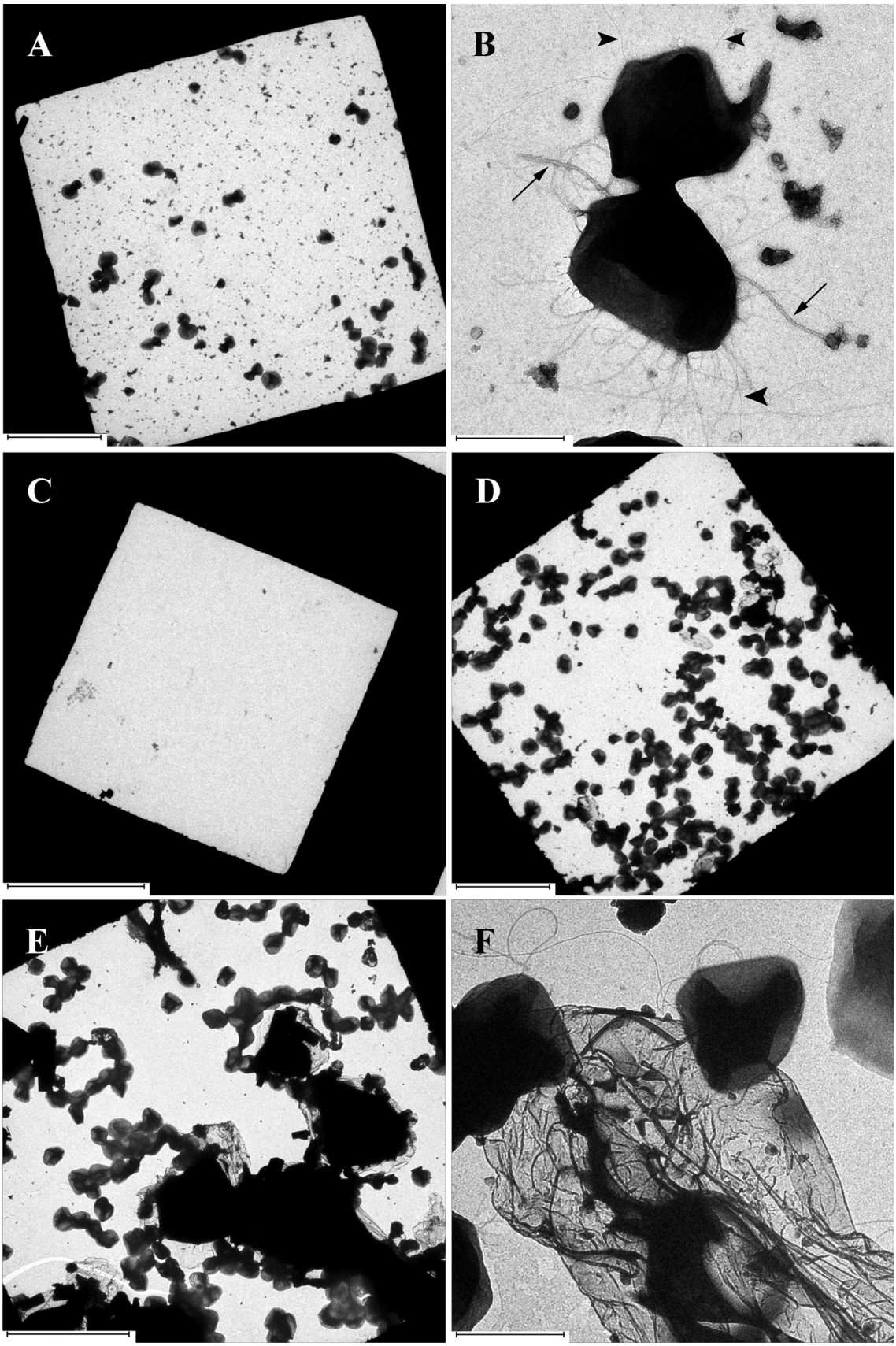

FIG. 1. Attachment of strain PBL2025 to carbon-coated gold grids. (A and B) Transmission electron micrographs of PBL2025 attached to carbon. (B) At higher magnifications, flagella (arrows) and pili (arrowheads) could be detected. (C) The Δ*flaJ* strain was not able to adhere under these conditions. When sulfur was added to the medium, considerably more PBL2025 cells attached to the carbon film (D), adhered to the sulfur particles (E), and produced an extracellular sheet-like material (F). Bars: 10 μm (A, D, and E), 1 μm (B and F), and 20 μm (C).

TABLE 2. Different materials tested for attachment of *Sulfolobus* strains

Surface	Growth of *S. solfataricus* strain			
	P2	PBL2025	Δ*upsE*	Δ*flaI*
Mica (glimmer)	Growth on surface with many flagella and pili	Growth on surface with pili	No growth	No growth
Glass	Growth on surface; cells are connected with a mesh of flagella/pili	Growth on surface; no pili or flagella visible	No growth	No growth
Pyrite (FeS_2)	Growth on surface	No growth	No growth	No growth
Gold grids coated with carbon	Growth on surface with pili and flagella	Growth on surface with pili and flagella	Only very few cells present	Only very few cells present

lipid metabolism (Table 3). Since the EPS produced by the two strains differed significantly, we explored whether the genes in the aforementioned region are involved in the production or modulation of EPS during growth of *Sulfolobales* on surfaces and whether the expression of the flagellin and pilin genes, the structural subunits of the flagella and the pili, respectively, is altered after attachment. Shaking *S. solfataricus* P2 cultures were incubated with pieces of mica for 2 days. Quantitative

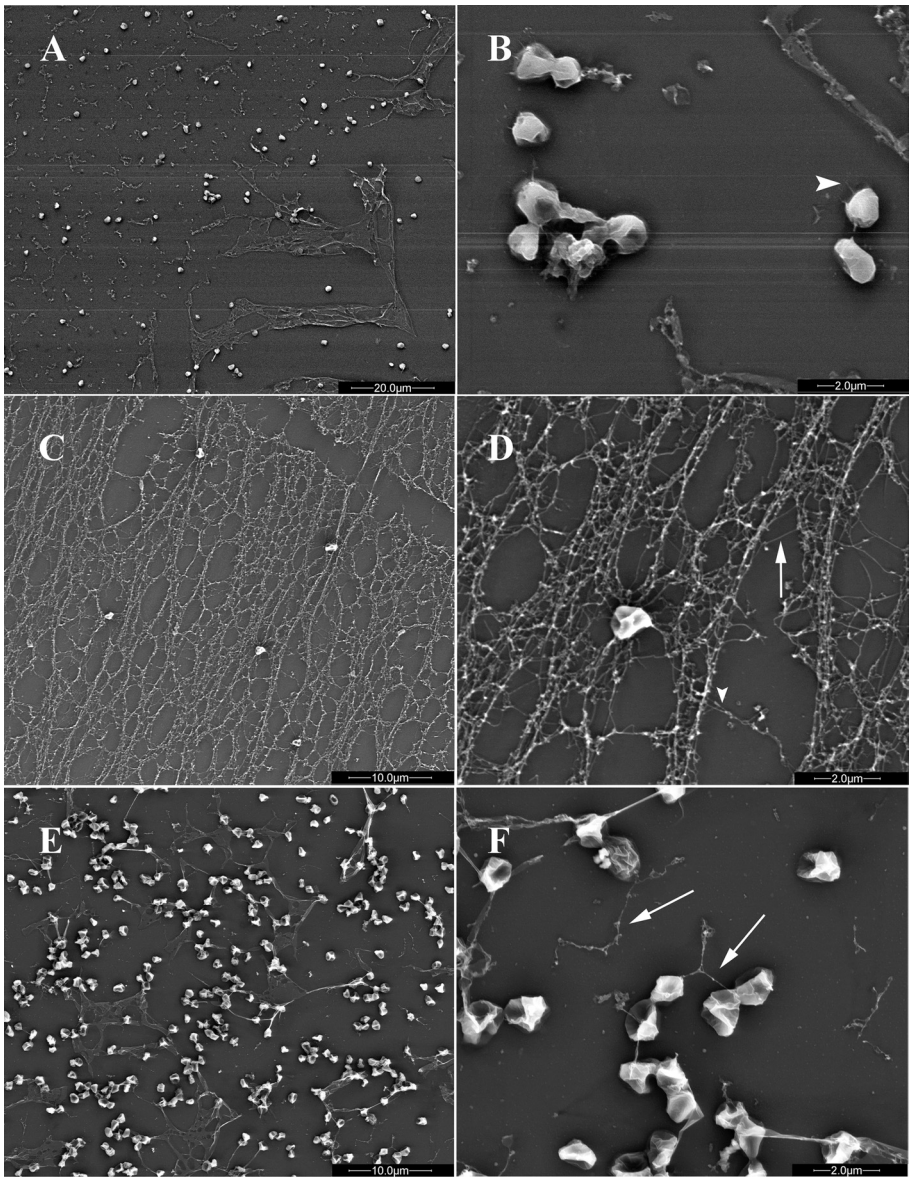

FIG. 2. Attachment of *S. solfataricus* P2 and PBL2025 to mica and glass. PBL2025 attached to mica and produced extracellular sheet-like structures (A and B), whereas P2 adhered via an extensive network of flagella/pili (C and D; panel D is an enlargement of panel C). Attachment of *S. solfataricus* P2 to glass was very different from that to mica (E and F; panel F is an enlargement of panel E). Pili (arrowheads) and flagella (arrows) are indicated. Bars: 20 μm (A), 2 μm (B, D, and F), and 10 μm (C and E).

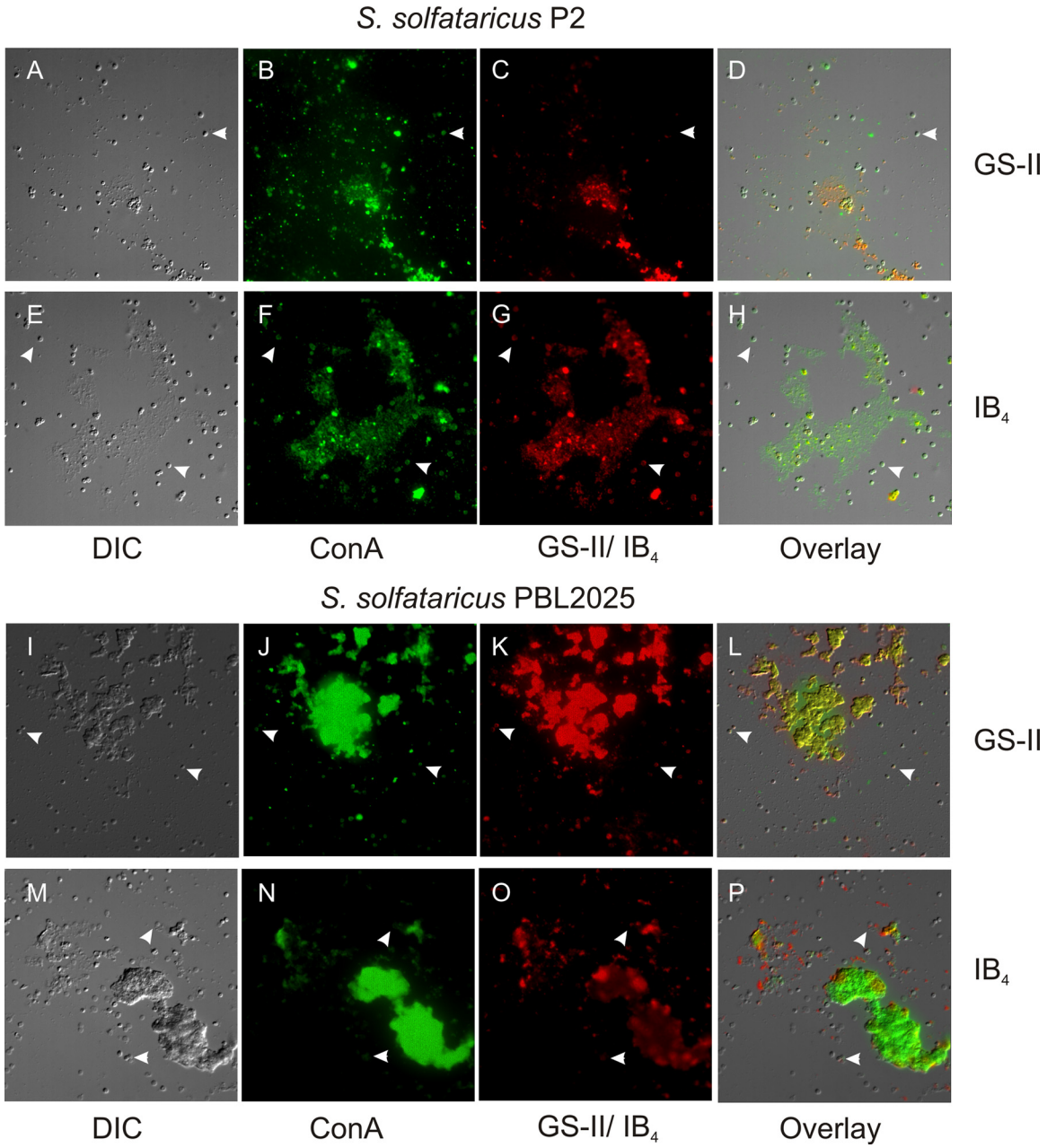

FIG. 3. Fluorescence microscopy using fluorescein-coupled ConA (green) and either Alexa-coupled GSII or IB4 lectin (both red) on *S. solfataricus* P2 (A to H) and *S. solfataricus* PBL2025 (I to P) attached to glass. (A, E, I, and M) Differential interference contrast; (B, F, J, and N) green channel; (C, G, K, and O) red channel; (D, H, L, and P) overlay of differential interference contrast and fluorescence images. White arrowheads indicate the positions of cells.

RT-PCR was performed on cDNAs obtained from total mRNAs isolated from planktonic and mica-attached cells.

Using qPCR, we determined that the expression of the flagellin FlaB was repressed 12-fold in attached cells in comparison to planktonic cells, whereas the UV-induced pilins, UpsA and UpsB, were upregulated 5- and 2-fold, respectively. Among the 18 tested genes in the genomic region of SSO3002 to SSO3050, 10 genes were not expressed in attached cells, whereas 8 were clearly induced (Fig. 4A and B). The genes induced strongly are predicted to be involved in sugar degradation and metabolism, such as a β-mannosidase (SSO3007); LacS, a β-galactosidase (SSO3019); two carbohydrate transporters (SSO3010/17); a glucose-1 dehydrogenase (SSO3009); and a gluconolactonase (SSO3041). An oxidoreductase (SSO3014) and a dihydrodipicolinate synthase (SSO3035), which is part of the lysine synthesis pathway, were induced as well.

DISCUSSION

In this study, we have shown that the hyperthermophilic archaea *S. solfataricus* P2 and PBL2025 are able to attach to a variety of surfaces, such as glass, mica, pyrite, and carbon-

TABLE 3. Putative functions of ORFs SSO3002 to SSO3048 and their induction patterns in attached cells

ORF	Putative function	Induction in attached cells[a]
SSO3002	Glycosyltransferase	–
SSO3003	Glucose 1-dehydrogenase	ND
SSO3004	3-Oxoacyl-(acyl carrier protein) reductase	ND
SSO3006	α-Mannosidase	–
SSO3007	Endo-β-mannanase	+
SSO3008	Dehydrogenase	ND
SSO3009	Carbon monoxide dehydrogenase, large chain	+
SSO3010	Carbohydrate transporter	+
SSO3011	Dehydrogenase	ND
SSO3012	ABC-type multidrug transporter	–
SSO3013	Dehydrogenase	ND
SSO3014	Oxidoreductase	+
SSO3015	Dehydrogenase	ND
SSO3016	Amino hydrolase	ND
SSO3017	Carbohydrate transporter	+
SSO3019	β-Glycosidase (LacS)	+
SSO3021	Zn-dependent hydrolase	–
SSO3022	α-Xylosidase	–
SSO3029	Sugar phosphate isomerase	ND
SSO3032	β-Xylosidase	ND
SSO3034	Hypothetical	ND
SSO3035	Dihydrodipicolinate synthase	+
SSO3036	β-Glucuronidase	–
SSO3037	Hypothetical	–
SSO3038	Hypothetical	–
SSO3039	Bile acid β-glucosidase	–
SSO3041	Gluconolactonase	+
SSO3042	Glucose dehydrogenase	–
SSO3043	ABC transporter, dipeptide binding protein	–
SSO3045	ABC transporter, ATP-binding protein	ND
SSO3046	ABC transporter, ATP-binding protein	ND
SSO3047	ABC transporter, permease	ND
SSO3048	ABC transporter, permease	ND

[a] ND, not determined.

coated gold grids, from shaking cultures, in a flagellum- and pilus-dependent manner. Cells lacking either the flagella or UV-inducible pili were unable to attach to the tested surfaces. The pili assembled by the *ups* operon so far have only been implicated in cellular aggregation after UV exposure. Our studies demonstrate that these pili are also expressed upon contact with surfaces and that there is an interplay with flagella in surface adhesion. Flagella have also been implicated in mediating surface adhesion and cell-cell contacts in the archaea *P. furiosus* and *M. kandleri* (12, 16), but our deletion mutant analysis demonstrated the requirement of these surface structures for attachment from shaking cultures. The qPCR data confirmed that the expression of the UV-induced pilins, UpsA and -B, was indeed upregulated in surface-attached cells. However, the expression of FlaB was drastically reduced in immobilized cells, from which the mRNA for the expression-level analysis was isolated after 2 days of incubation. Most probably the flagella are necessary for initial attachment and possibly "recognition" of surfaces, but not for persistence once the cells are attached.

PBL2025 formed extensive sheets of extracellular material, whereas *S. solfataricus* P2 synthesized an extensive network of flagella and pili in order to attach to glass and mica. We utilized fluorescent lectins to study the composition of the EPS formed by these two strains. Glucose, α-D-mannose, α-D-galactose, and *N*-acetyl-D-glucosamine are the minimal components of EPS, since a selective reaction was observed with ConA, GS-II, and isolactin-IB4 (Fig. 3). This sugar composition matches the sugars found in extracellular glycoproteins of *S. solfataricus* and EPS isolated from shaking *S. solfataricus* MT4 cultures (7, 13). We could not observe flagella and Ups

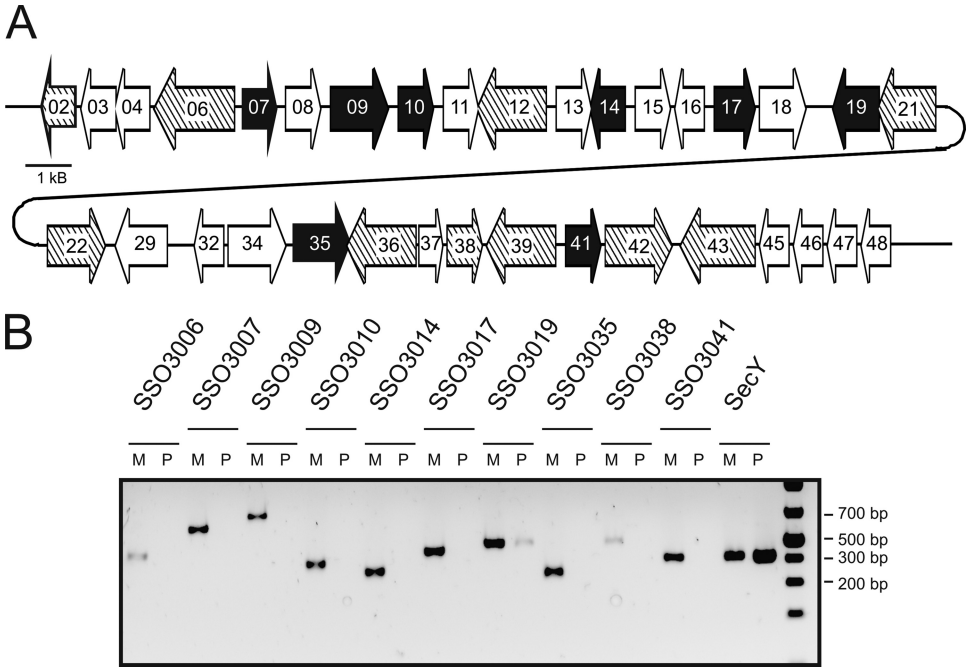

FIG. 4. Expression analysis of the genomic region SSO3002 to SSO3048. (A) Schematic view of the genomic region SSO3002 to SSO3048. Genes indicated by hatched arrows were not expressed in attached cells, whereas filled black arrows indicate genes induced under attached conditions. (B) Agarose gel of RT-PCRs performed on cDNAs from planktonic cells (P) and mica-attached cells (M). *secY* was used as a quality control for the isolated RNA and as a housekeeping gene.

pili on mica and glass as clearly as in the transmission electron micrographs from carbon-coated grids. A possible explanation is that both flagella and Ups pili are structural components within the EPS network. A recent study on surface attachment of an *Escherichia coli* K-12 strain to glass demonstrated that α-D-mannose-rich EPS structures on glass surfaces are essential for lateral biofilm maturation of the cells (14). The EPS coat on the hydrophilic glass creates a template for the hydrophobic type 1 fimbria filaments of *E. coli* and subsequent development of biofilm. In the case of *S. solfataricus*, the EPS was produced in large amounts during the attachment of cells to mica and glass, while on carbon-coated grids similar EPS structures were absent. Both glass and mica surfaces are hydrophilic, and the carbon films on electron microscopy grids are hydrophobic. One possible explanation is that *S. solfataricus* EPS creates a template on a hydrophilic surface which is more suitable for other cell surface structures, such as the S-layer envelope or other glycosylated components of the cell surface, during the following development of cells.

S. solfataricus P2 and PBL2025 differed in the amount and form of the EPS secreted after 3 days of surface attachment. Since PBL2025 lacks a quite large genomic region present in *S. solfataricus* P2, we demonstrated that at least 10 of these genes were upregulated upon surface attachment. The majority of these genes are predicted to be involved in sugar degradation and transport. These proteins might be involved in the efficient degradation of the produced EPS or in its modification and modulation, which might explain why with PBL2025 an extensive surface labeling of the EPS was observed. Among the upregulated proteins was LacS, a very-well-characterized β-galactosidase, and its cognate lactose transporter, which have both been shown to be essential for *S. solfataricus* for growth on lactose (3). Since we detected glucose and galactosyl residues by lectin staining, lactose might be present as a breakdown product of the EPS. Studies on bacterial EPS in biofilm suggest that glycosylation functions as a means for nutrition storage (4, 6, 9, 11, 18). Therefore, it will be interesting to determine the purpose of EPS in archaea and whether it is composed only of sugars or contains proteins besides the flagellins and pilins. We have demonstrated that flagella and UV-induced pili are essential for initial attachment of *S. solfataricus*, and it will be vital to determine which role they play in the development and consolidation of biofilms. Future gene inactivation studies will therefore focus on the role of surface structures, EPS production, and degradation in this process.

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